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Direct preparation of cyclodextrin monophosphates

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Abstract

Aqueous solutions of cyclodextrins and inorganic metaphosphate at pH 4, upon drying and subsequent warming, produce mixtures of isomeric monophosphate esters which are amenable to separation by anion-exchange chromatography. The products are characterised by enzymatic, mass, and NMR spectroscopic analysis. The methodology provides a route to these derivatives by a single reaction. © 1997 Elsevier Science Ltd. © 1997 Elsevier Science Ltd.

Keywords: Cyclodextrins; Phosphate; Esters; Metaphosphate; Direct preparation

1. Introduction

Cyclodextrins are cyclic oligosaccharides possessing, in the main, 6, 7, or 8 $\alpha(1 \rightarrow 4)$ glucopyranose residues [1]. The molecules adopt toroidal shapes with secondary hydroxyls occupying the wider side of the cone, primary hydroxyls the narrower side, and the cavities comprising H-atoms and glycosidic oxygens. As a result, the outer surface is hydrophilic, conferring solubility in aqueous media, whilst the cavities are hydrophobic and are able to accommodate and complex some organic molecules. Such inclusion complexes find applications in microencapsulation of sensitive compounds related to food and pharmaceuticals and as chiral agents capable of enhancing enzyme–substrate and drug–receptor interactions [2–4]. Recent reports have also noted their use as stationary phases for chiral analysis and purification procedures [3,5,6]. Replacement of hydroxyls with other functional groups has been shown to improve remarkably the ability of cyclodextrins to form inclusion complexes and enhance catalytic properties, and the preparation of various cyclodextrin derivatives has been reported [7]. A number of research reports concerned with cyclodextrins have also been published in a special issue of *Carbohydrate Research* [8].

We have recently shown [9–13] that compounds containing primary and/or secondary alcohol groups undergo phosphorylation when dried in the presence of inorganic phosphate salts. Increased rates and extents of phosphorylation are observed with metaphosphate, and the reaction occurs over a range of pH (below 3 to above 8), but is favoured under acidic conditions, being optimal around pH 4. The methodology has provided *direct* routes to a number of disaccharide monophosphate esters, and this report

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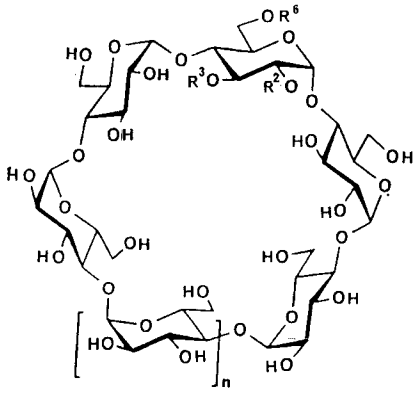
describes results obtained using α-cyclodextrin (αCD), **1**, and β-cyclodextrin (βCD), **2**.

2. Results and discussion

After drying in the presence of metaphosphate at pH 4 and subsequent heating at 90 °C, αCD and βCD each produced three major products with retention times on high-pH anion-exchange chromatography (HPAEC) expected from monophosphate esters. The products are designated **A**, **B**, **C** and **D**, **E**, **F** (Fig. 1a,b). γ-Cyclodextrin, **3**, produced a similar mixture but has not been investigated further. It is noteworthy that αCD was highly reactive (products were observed without heating), and this reactivity contrasts markedly with all other polyols which we have studied so far. For mixtures which were heated for longer periods, chromatographically more strongly retained products were observed and these are probably polyphosphorylated species.



1: $R^2 = R^3 = R^6 = H$; n = 1
2: $R^2 = R^3 = R^6 = H$, n = 2
3: $R^2 = R^3 = R^6 = H$, n = 3
A: $R^2 = R^6 = H$, $R^3 = PO_3M_2$, n = 1
B: $R^3 = R^6 = H$, $R^2 = PO_3M_2$, n = 1
C: $R^2 = R^3 = H$, $R^6 = PO_3M_2$, n = 1
D: $R^2 = R^6 = H$, $R^3 = PO_3M_2$, n = 2
E: $R^2 = R^3 = H$, $R^6 = PO_3M_2$, n = 2
F: $R^3 = R^6 = H$, $R^2 = PO_3M_2$, n = 2
$M = NH_4^+$ or K^+

Fractionation of reaction mixtures on AG1 anion-exchange resin, as used previously [12,13] for the isolation of other oligosaccharide phosphates, resulted in poor resolution because of slow leaching from the resin of the organic components present in

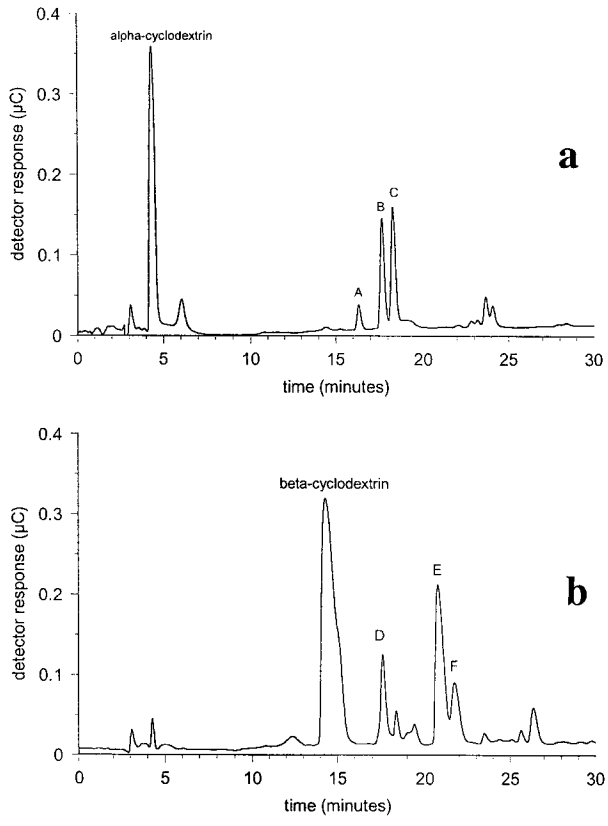


Fig. 1. HPAEC chromatogram for (a) αCD and metaphosphate, and (b) βCD and metaphosphate. For details see Experimental.

the mixture. This occurred even at high (1.5 M) salt concentrations indicating that interactions additional to ionic were operative. These interactions could result from guest–host complexation [1,14] between aromatic residues of the styrene–divinyl benzene resin matrix and the hydrophobic cavities of the cyclodextrin molecules, and in keeping with this the fractionation on QAE Sepharose (which is dextran based and should therefore not complex) resulted in satisfactory resolution of the products and their separation from inorganic phosphate (P_i). Using this latter resin, **C** and **E** were isolated as major components in either case, and total yields, based upon the recovery of organic phosphorus as monophosphate esters, were 17% from αCD and 10% from βCD. Further fractionation of the products by preparative HPAEC followed by ion- and gel-filtration provided analytical samples of the individual components. Their structures were determined using a combination of elemental, enzymatic, mass, and NMR analysis. The organic phosphorus-containing fractions after separation from P_i contained [15] 2.8% w/w P (from αCD) and 2.3% w/w P (from βCD), in keeping

with the presence of one phosphorus per molecule. Incubation of these mixtures with phosphomonoesterase converted more than 90% of the organic phosphorus to P_i, simultaneously liberating αCD (from **A**, **B**, **C**) or βCD (from **D**, **E**, **F**), confirming that the products are α- and β-cyclodextrin orthophosphate esters.

The FAB mass spectra of **A**, **B**, and **C** (K^+ salts) all contained a major ion at *m/z* 1091.2 corresponding to the molecular ion of αCD mono-orthophosphate (calculated 1091.5 for the protonated potassium hydrogen species, $C_{36}H_{61}O_{33}PK$). Major ions were also present at *m/z* 1053 and 1129 corresponding, respectively, to the dihydrogen and dipotassium species. Ions at higher values of *m/z* were not observed, indicating that each product contained only a single phosphate. The FAB mass spectra of **D**, **E**, and **F** all contained major ions at *m/z* 1291.5 corresponding to molecular ions of βCD mono-orthophosphate (calculated 1292.1 for the protonated dipotassium species, $C_{42}H_{70}O_{38}PK_2$). Ions were also observed at *m/z* 1215 and 1253 corresponding, respectively, to the dihydrogen and potassium hydrogen species. These results indicate that **D**, **E**, and **F** also contain only a single phosphate.

Monophosphorylation could be confirmed and its position of substitution determined from the 1D and TOCSY [16,17] ^1H NMR spectra. Monosubstitution of a cyclodextrin results in the loss of molecular symmetry, and the spectra of such derivatives are consequently more complex than those of the parent oligosaccharides in which all residues appear equivalent. The spectra obtained from the products could, however, be approximated to a composite of two sets of resonances, one from the unsubstituted glucopyranosyl residues, the other from a phosphorylated residue. Most of the protons from the unsubstituted residues appeared at chemical shifts close to those of

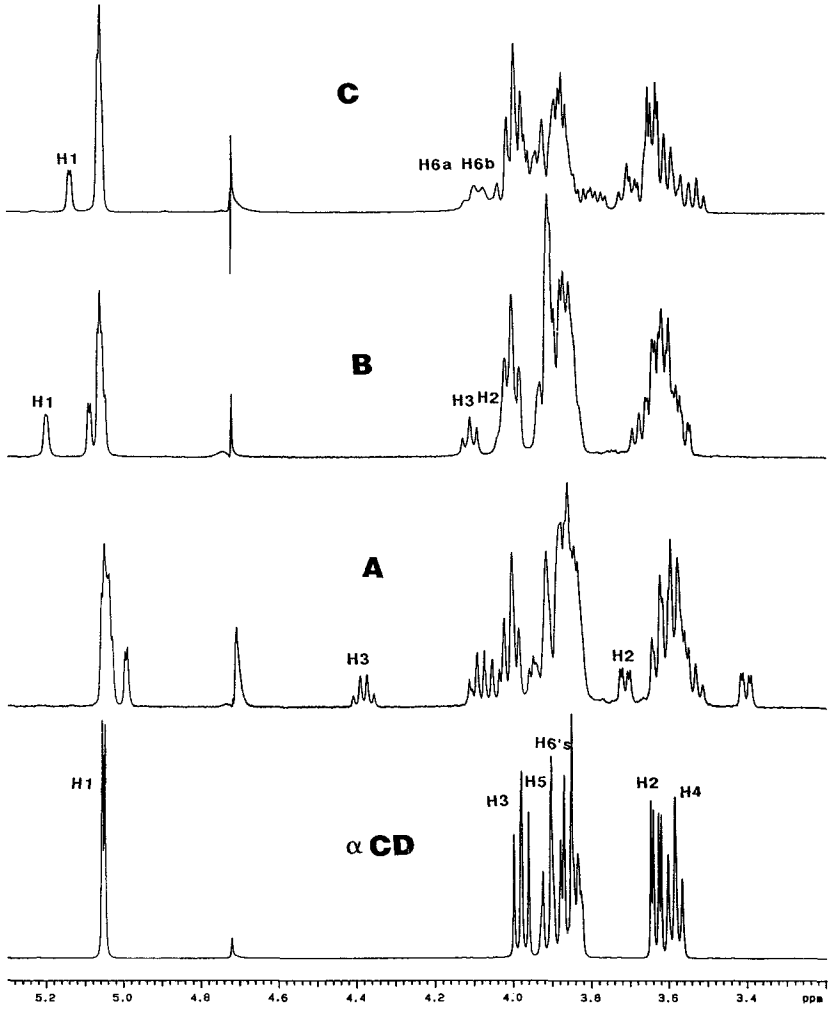


Fig. 2. ^1H NMR spectra of αCD and **A**, **B**, **C**. Protons of the phosphorylated residues of **A**, **B**, and **C** are indicated.

the parent cyclodextrin, whereas protons present in the phosphorylated residue were shifted to lower field. From the shifts of the latter, the position of the phosphate group could be ascertained since geminal protons are perturbed by up to +0.55 ppm and vicinal protons by up to +0.2 ppm [12,18]. In some instances protons in non-phosphorylated residues were also perturbed, apparently as a result of inter-residue affects of the phosphate group.

The spectra of **A**, **B**, and **C** together with that of αCD are shown in Fig. 2, and the observed chemical shifts and correlated resonances (from TOCSY spectra) are listed in Table 1. For **A**, a one-proton quartet (δ 4.39, $J \sim 9$ Hz) characteristic of H-3 in a glucopyranosyl-3-phosphate residue [12,13,18] was present, and the TOCSY spectrum showed this quartet to be associated with resonances at δ 5.05, 3.84, 3.72, and 3.62 which can be assigned to H-1, H-5, H-2, and H-4 of the same residue, respectively. The chemical shift perturbations, relative to αCD, of especially H-3 (+0.41 ppm) and H-2 (+0.08 ppm) are as expected from the presence of a 3-phosphate group, and the data are in accordance with α-cyclodextrin-3-phosphate. It is noteworthy that additional TOCSY correlations are observed (see [a] in Table 1), and these resonances can be assigned to H-1, H-2, H-3, and H-4 of a non-phosphorylated residue with its H-4 displaced *upfield* by 0.23 ppm relative to its chemical shift in the parent saccharide. This would appear to result from the influence of the phosphate group present in a *different* residue; similar affects have previously been observed across residues in other oligosaccharide phosphates [12,13].

For **B**, anomeric protons were present at δ 5.02 (four protons), 5.05 (one proton), and 5.17 (one proton). The resonance at 5.17 was associated with resonances at δ 4.09, 3.99, and 3.66, and these can be assigned, respectively, to H-1, H-3, H-2, and H-4 of a glucopyranosyl-2-phosphate residue. The perturbations of these resonances, from their values in αCD, are +0.11, +0.11, +0.35, and +0.07 ppm and are in accordance with the presence of a 2-phosphate group [12,13,18], and **B** is therefore assigned as α-cyclodextrin-2-phosphate. For **C** a two-proton multiplet at δ 4.08 is present as would be expected from the *O*-phosphorylated methylene group, and this resonance was associated with those at δ 3.92 and 3.69 which can be assigned to H-5 and H-4, respectively. These shifts are similar to corresponding protons in the 6-phosphates of trehalose, sucrose, and lactose [12,13,18], and **C** is therefore assigned as α-cyclodextrin-6-phosphate. Resonances marked [a] in Table 1 are associated protons in a non-phosphorylated residue with H-1 and H-4 exhibiting inter-residue affects similar to those observed for **A**.

In a like manner, **D**, **E**, and **F**, derived from βCD, were identified from their NMR spectra which, together with the spectrum of βCD, are shown in Fig.

Table 1
^{1}H chemical shifts (ppm from Me_4Si) in D_2O at 500 MHz and 30 °C of α-cyclodextrin and **A**, **B**, **C** (K^+ salts)

H	αCD	**A** (3-phosphate)		**B** (2-phosphate)		**C** (6-phosphate)	
1	5.06	5.00 [a] [1] [b]	**5.05** [c,d] [5]	5.02 [4] 5.05 [1]	**5.17** [d] [1]	5.13 [a] [1]	**5.06** [5]
2	3.64	3.41 [a] [1] 3.63	**3.72** [d] [1]	3.62	**3.99** [d,e]	3.62 [a] 3.71 [1]	
3	3.98	4.01 [3] 4.07 [a] [1] 4.10 [1]	**4.39** [d] [1]	3.98	**4.09** [d,e]	3.98 [a]	
4	3.59	3.39 [a] [1] 3.58	**3.62** [d,e]	3.58	**3.66** [d,e]	3.52 [a] [1] 3.60	**3.69** [d,e]
5	3.72–3.84	3.82–3.97	**3.84** [d,e]	3.71–3.92		3.72–3.94	**3.92** [d,e]
6a,b	3.72–3.84	3.82–3.97		3.71–3.92		3.72–3.94	**4.08** [d] [2]

[a] Associated resonances in a non-phosphorylated residue from TOCSY.
[b] Number of protons from integration.
[c] Chemical shifts in bold are protons in the phosphorylated residue.
[d] Associated resonances in the phosphorylated residue from TOCSY.
[e] Chemical shift from TOCSY.

3, with their chemical shifts and correlation data set out in Table 2. The spectrum obtained from **D** was very similar to that of **A** and, in particular, it contained a one-proton quartet at δ 4.37, characteristic for H-3 of a glucopyranosyl-3-phosphate residue. Product **D** is therefore assigned as β-cyclodextrin-3-phosphate. The spectrum of **E** contained a two-proton multiplet at δ 4.07, in keeping with an *O*-phosphorylated methylene group, and on the basis of this and its correlated spectrum, **E** was assigned as β-cyclodextrin-6-phosphate. The spectrum of **E** was, as expected from this assignment, very similar to that of **C**, even to the extent of exhibiting inter-residue affects for a H-1 and H-4 as indicated in Table 2. For **F**, its spectrum closely resembled that obtained from **B** with the lowest field anomeric proton (δ 5.21) associated with a two-proton multiplet at δ 4.06 (H-2 and H-3). These values are as expected from β-cyclodextrin-2-phosphate and **F** is therefore assigned this structure.

In all of the spectra, all other protons could be accounted for and (except for those affected by inter-residue effects as noted) were observed at or near their chemical shift values in the parent saccharide.

Many phosphate esters are biochemicals, but to our knowledge cyclodextrin phosphates are not found in Nature, nor has the preparation of α-cyclodextrin phosphates been described. β-Cyclodextrin monophosphates have been synthesised earlier [19] via selective phosphorylation (the 6-isomer) and result as products (the 2- and 3-isomers) when bis(*m*-nitrophenyl) phosphate is hydrolysed in the presence of βCD with which it complexes. αCD is, as mentioned, considerably more reactive under the phosphorylating conditions than βCD or other polyols.

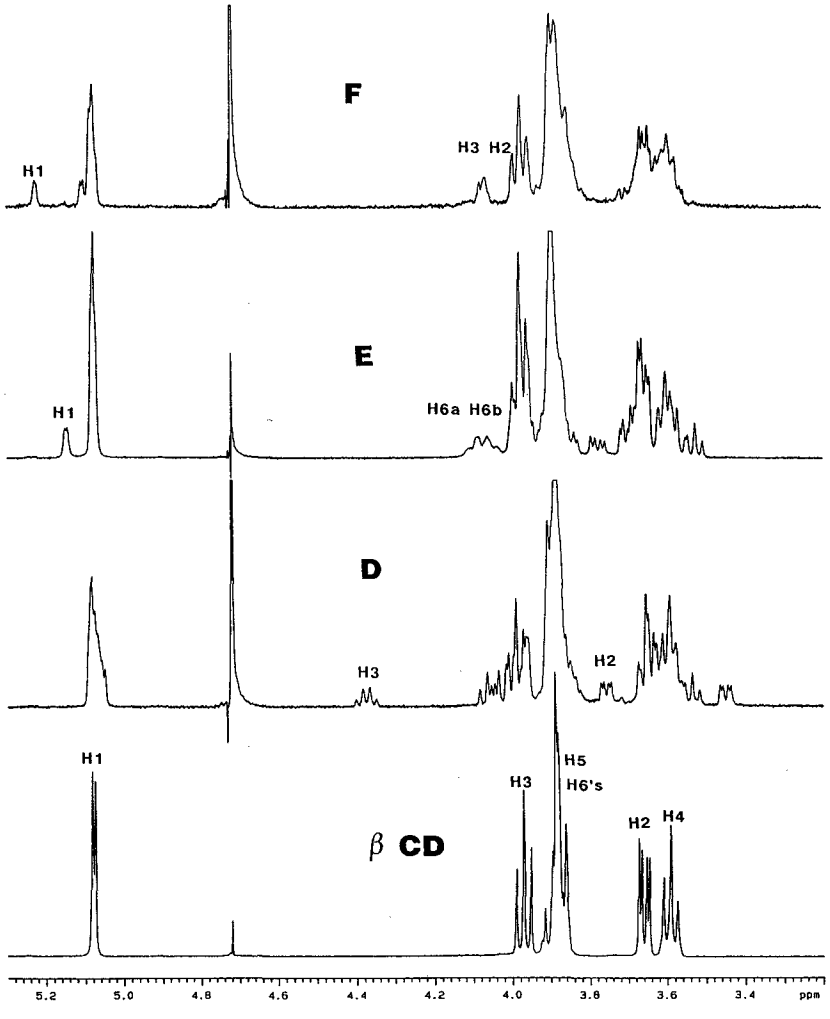


Fig. 3. ^{1}H NMR spectra of βCD and **D**, **E**, **F**. Protons of the phosphorylated residues of **D**, **E**, and **F** are indicated.

Table 2
^{1}H chemical shifts (ppm from Me_4Si) in D_2O at 500 MHz and 30 °C of β-cyclodextrin and **D**, **E**, **F** (K^+ salts)

H	βCD	**D** (3-phosphate)		**E** (6-phosphate)		**F** (2-phosphate)	
1	5.09	5.39 [a] [1] [b]	**5.06** [c,d] [6]	5.14 [a] [1]	**5.07** [d] [6]	5.06 [5] 5.10 [a] [1]	**5.21** [d] [1]
2	3.65	3.44 [1] 3.65	**3.75** [d] [1]	3.67		3.65 [a]	**4.06** [d] [2]
3	3.97	3.97 [a] 4.02 [1] 4.05 [1]	**4.37** [d,e] [1]	3.97 [a]		3.97	**4.06** [d] [2]
4	3.59	3.53 [1] 3.60 [a]	**3.65** [d,e]	3.51 [a] [1] 3.60	**3.69** [d,e]	3.58	
5	3.82–3.92	3.80–3.93		3.74–3.96	**3.96** [d,e]	3.80–3.92	
$6_{a,b}$	3.82–3.92	3.80–3.93		3.74–3.96	**4.07** [d] [2]	3.80–3.92	

[a] Associated resonances in a non-phosphorylated residue from TOCSY.
[b] Number of protons from integration.
[c] Chemical shifts in bold are protons in the phosphorylated residue.
[d] Associated resonances in the phosphorylated residue from TOCSY.
[e] Chemical shift from TOCSY.

This may be the result of the association of αCD with the phosphorylating species; anion complexes are reported e.g. for acetate [20], halide, isocyanate, chlorate, and others [14]. The nature of any association in the present case is, however, not clear. Preliminary results obtained from titrating αCD with up to one equivalent of sodium metaphosphate did not, for example, cause any significant changes in the ^{1}H NMR spectrum as would have been expected if a guest–host anionic complex had formed with a major anionic species. The observed high reactivity of αCD may nevertheless provide useful information on the nature of the reacting species and reaction mechanism of the phosphorylation under 'dry' conditions which are not yet known in detail.

To summarise, a single phosphate ester can be directly introduced into cyclodextrins by reaction with inorganic phosphate-producing mixtures containing the three possible isomers which are separable by anion-exchange chromatography. The reaction conditions have not been optimised for maximum yields, and variable amounts of products have been observed, by chromatograpic analysis, possibly as a result of different residual moisture contents of different preparations after drying. αCD has undergone significant phosphorylation (1–2%) immediately following drying at room temperature, with the reaction reaching equilibrium after a few hours at elevated temperature and βCD reaching equilibrium only after several tens of hours. For both, yields of 2-, 3-, and 6-isomers are in the approximate ratio of 1:1:3, i.e. the less sterically hindered primary hydroxyls esterify about 3 times more readily than the secondary hydroxyls. Although the conversions reported here are not particularly high, there are distinct advantages over other methods of preparation as the phosphates are prepared by a single reaction using low-cost phosphorylating reagents and the starting substances can be readily recycled adding to the overall efficiency of the process.

3. Experimental

General methods.—α- and β-Cyclodextrins were obtained from Sigma (Poole, UK), QAE 25 Sepharose from Pharmacia (Uppsala, Sweden). ^{1}H NMR spectra were determined under ambient conditions on solns of potassium salts in D_2O at 500 MHz on a Varian Unity Spectrometer. TOCSY spectra were determined with a mixing time of 60 ms. HPAEC was performed on a Dionex BioLC system with pulsed amperometric detection using a PA1 (4 × 250 mm) column for analytical work and a PA1 (9 × 250 mm) column for preparative work and, unless otherwise indicated, using a mobile phase of 100 mM NaOH with a NaOAc gradient (100–160 mM, over 25 min). Fast-atom bombardment (FAB) mass spectra were recorded on a Kratos MS 80 RFA Mass Spectrometer equipped with an Ion-Tech saddle field atom gun and standard Kratos FAB source (Kratos Analytical, Manchester, England). Xenon atoms were used as the bombarding particles and the liquid matrix was a 1:1 mixture of glycerol and a eutectic mixture of dithiothreitol and dithioerythritol (5:1). Approximately 3 μg of sample dissolved in 1 μL 30% CH_3COOH was mixed with

an equal vol of matrix on the stainless steel target. The instrument was operated at 4 kV accelerating voltage and the magnet was scanned at 10 s/decade over a mass range of 1500 to 100. Masses were assigned, in the positive mode, by comparison with caesium iodide clusters. Approximately 10 scans were acquired and averaged using the raw data program supplied with the DS90 data system.

For other methods see Ref. [12]

α-Cyclodextrin 3-, 2-, and 6-phosphates (**A, B** and **C**).—α-Cyclodextrin **1** (1.0 g) was dissolved in 1 M sodium metaphosphate (10 mL, pH 4) and the soln was freeze-dried. The container was then sealed and heated at 90 °C for 6 h. HPAEC analysis of the reaction mixture indicated the formation of three major products eluting after α-cyclodextrin (Fig. 1a). The white residue was dissolved in distilled water (100 mL) and the soln loaded onto an anion-exchange QAE 25 Sepharose column (2.5 × 50 cm) in the acetate form which was eluted with a linear gradient (0.2–1 M) of ammonium acetate. The organic phosphorus-containing fractions which eluted before P_i were freeze-dried (to constant weight) to sequentially give **C**, **A**, and **B** (which were only partially resolved) as white solids. These products contained 2.7% by weight phosphorus (calculated for α-cyclodextrin monophosphates ammonium salts, P 2.85%). The amount of organic phosphorus (4.51 mg) in these fractions corresponds to a total yield of 175 mg (17%) of α-cyclodextrin monophosphates, half of which was **C**. Appropriate fractions from the QAE separation (in 10 mg quantities) were then fractionated on a CarboPac PA-1 (9 × 250 mm) column using a gradient of 100–280 mM NaOAc in 100 mM NaOH over 40 m and a flow rate of 2 mL/min. Fractions were collected after they had passed through a membrane suppresser (Dionex), in order to exchange sodium ions, and were inspected using the analytical HPAEC system. Fractions containing the individual components were combined and desalted on a column of polyacrylamide P2 (BioRad, 42 × 1.5 cm) and converted to their potassium salts (simultaneously removing any multivalent cations) by passage through a column of Chelex 100 (BioRad, K^+ form) column (12 × 1 cm). The solns were then filtered and freeze-dried to give analytical samples of **A**, **B**, and **C** as white amorphous solids which are, respectively, the 3-, 2-, and 6-phosphates of α-cyclodextrin.

β-cyclodextrin 3-, 6-, and 2-phosphates (**D, E** and **F**).—β-Cyclodextrin **2** (1.0 g) was dissolved in 0.1 M sodium metaphosphate (100 mL, pH 4) and the soln freeze-dried. The container was then sealed and heated for 7 days at 90 °C. HPAEC of the reaction mixture indicated the formation of three major products eluting after β-cyclodextrin (Fig. 1b). The residue in water (100 mL) was then fractionated on QAE 25 Sepharose as described above to give three organic phosphorus-containing fractions corresponding to, in order of elution, **E** and partially resolved **D** and **F**. These contained 2.3% w/w phosphorus (calculated P 2.48% for β-cyclodextrin monophosphate ammonium salts). The total organic phosphorus (2.5 mg) in these fractions corresponds to a total of 101 mg (10%) of β-cyclodextrin monophosphates. Further fractionation on the preparative PA 1 column with a gradient of 200–550 mM NaOAc, followed by desalting and conversion to potassium salts was carried out as described above. In this way the β-cyclodextrin monophosphates **D**, **E**, and **F** which are, respectively, the 3-, 6-, and 2-isomers were obtained as white amorphous solids.

Digestion with alkaline phosphatase.—Each product (0.5 mg) was treated with alkaline phosphatase (5 units) in aq 0.05 M $(NH_4)_2CO_3$, 0.01% NaN_3, pH 8.2 (1 mL) at 37 °C and the solns assayed for total and inorganic phosphorus. Control samples lacking enzyme were also prepared and similarly assayed. The reaction mixtures were also analysed by HPAEC.

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